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RESEARCH ARTICLE

Impact of Potential Fermentation Inhibitors Present in Sweet Sorghum Sugar Solutions

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Abstract In this work, the fermentation of the sweet sorghum sugars such as sucrose, glucose, and fructose to ethanol was studied in the presence of acetic acid, lactic acid, and aconitic acid, which are present in the juice or produced by microorganisms during prolonged storage of harvested materials or juice. An industrial strain of distiller's yeast was used to produce ethanol from 100 g/L (83 g/L after inoculum) of total sugars. The fermentation time ranged from 12 to 140 h, with the longer fermentation time corresponding to clear inhibition of yeast growth and product accumulation in the presence of 8 g/L of initial acetic acid. Among the acids, only acetic acid showed a negative impact on the fermentation rates and only at levels greater than 2 g/L. Lower levels of acetic acid and all levels of lactic acid and aconitic acid (1–5 g/L) either showed an improvement in fermentation rates or in final ethanol concentration. The acidity was not controlled during the fermentation but was initially adjusted, and it is presumed that the pH buffering effect on the organic acids contributed to the higher fermentation rates and prevented the pH from naturally dropping as the fermentation progressed.

Keywords Acetic acid · Lactic acid · Aconitic acid · Ethanol · Biofuel

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Introduction

Lignocelluloses are a non-food resource for the production of biofuels and chemicals via fermentation. The initial step requires pretreatment in the form of hydrolyzing the carbohydrates into fermentable sugars. During pretreatment, sugar and lignin degradation products are produced that are inhibitory to many microorganisms and may prevent fermentation, extend the lag phase, and/or reduce product yield (Boyer et al. 1992). The main inhibitors are furfural (mainly from xylose degradation) and hydroxymethylfurfural (mainly from glucose degradation). These inhibitors must be removed or controlled in order to optimize the fermentation yield, production rate, and/or titer (Klinke et al. 2004; Huang et al. 2008; Klasson et al. 2013). Just as in the case of lignocellulosic sugars, the processing of other non-food sugars may result in the generation of fermentation inhibitors. In sugar recovery from sweet sorghum, fermentation inhibitors (such as aconitic acid) are sometimes naturally present in the juice (Eggleston et al. 2010; Wu et al. 2010) or organic acids (e.g., acetic and formic) are generated from glucose and fructose degradation during evaporation to create storable syrup for subsequent fermentation (Eggleston et al. 2013). In addition, short-chain organic acids may be generated during non-aseptic storage of juice or stalks by the action of hetero- and homo-fermentative bacteria (Wu et al. 2010) and preservation/storage of sweet sorghum juice is needed for effective processing of juice (Kumar et al. 2013; Eggleston et al. 2015). Among inhibitors to fermentation, organic acids have experimentally been removed via over-liming (and filtration), by ion-exchange or adsorption, or by overcoming the inhibition by higher cell concentrations (Boyer et al. 1992; Mussatto and Roberto 2004). Phenolics often associated with color in sugar crop juice are removed by

activated carbon treatment (Mussatto and Roberto 2004). Phenolics and furans can also be converted by microbial fuel cells generating electricity (Borole et al. 2009).

Sweet sorghum (*Sorghum bicolor* L. Moench), originally from Africa, has been identified as a potential bioenergy crop as it can produce starch (in grain), sugar (in stalk juice), and fibrous biomass. It is a fast-growing, resistant to both biotic and abiotic pressures, and efficient CO_2 user with less need for fertilizer than sugarcane (Prasad et al. 2007; Almodares and Hadi 2009; Wu et al. 2010; Kim and Day 2011; Serna-Saldivar and Rooney 2014). The juice from sweet sorghum contains higher levels of invert sugars than sugarcane (or energy cane) juice (Kim and Day 2011), making it less suitable for crystalline sugar manufacture but useful for biological conversion to biofuels and bioproducts.

The work described within investigated the impact of potential inhibitors, such as acetic acid, lactic acid, and aconitic acid, on the fermentation of sweet sorghum juice sugars to bioethanol by yeast.

Materials and Methods

Fermentation of sugars was carried out in 250-mL ANKOM (Macedon, NY) fermentation bottles equipped with pressure monitoring and control in order to measure the amount of CO_2 produced. Experiments were performed by starting a culture from dry yeast cells (Red Star Distiller's Active Dry Yeast, BSG Handcraft, Shakopee, MN) by adding 1 g of yeast to 19 mL of activation broth, containing glucose (5 g/L), Bacto peptone (5 g/L), Bacto yeast extract (3 g/L), KH_2PO_4 (1 g/L), and $MgSO_4{\cdot}7H_2O$ (0.5 g/L) in deionized water and adjusted to pH 4.5 with 0.1 M HCl. The activation was carried out at 32 °C for 30–40 min, and then, the entire activation culture (approx. 20 mL) was added to each of the fermentation bottles with the growth/production medium. The synthetic 100 mL growth/production medium contained sucrose (60 g/L), glucose (25 g/L), fructose (15 g/L), and (in most cases) inhibitors in deionized water and adjusted to pH 4.5. For example, in the case of acetic acid, ten fermentation bottles were used [including one control with distilled water as production medium] containing yeast, activation broth (approx. 20 mL), and production medium with sugars and nine different levels of acetic acid (0–8 g/L).

Each bottle contained one glass marble (to aid in mixing) and three drops Antifoam A Concentrate (Sigma-Aldrich, St. Louis, MO). The head space ($\sim$190 mL) was flushed with helium (200 mL/min) for 3 min, and the bottles were placed in a shaking (175 rpm) incubator at 30 °C. Strict sterile techniques were not used; however, synthetic solutions were filter sterilized (2 µm-pore-size filters) and fermentation bottles were rinsed with ethanol (70 % v/v) as sanitizing agent.

At the time of sampling (usually at the end of the fermentation), 1.5 mL of cell broth was added to pre-weighed 2-mL centrifuge vials and centrifuged at 9500*g* for 5 min. The supernatant was removed and filtered through a syringe filter (0.2-µm nylon) for analysis. The cell pellet was re-suspended and washed with 1.5 mL deionized water and centrifuged again. The washing step was repeated and the supernatant was removed, and the vials were dried at 100 °C, overnight, before the dry cell weight (DCW) was determined. The DCW procedure was done in triplicate.

Analysis of sugars and products was carried out by high-performance liquid chromatography with a refractive index detector (Series 1100 Hewlett Packard/Agilent, Santa Clara, CA). The mobile phase was 5 mM H_2SO_4, which was pumped at 0.6 mL/min through an Aminex HPX-87H column with a Cation H guard column (Bio-Rad, Hercules, CA). Samples were injected (2–10 µL) twice; once for sugar analysis with the column held at 20 °C to prevent hydrolysis of sucrose and secondly with the column held at 50 °C for analysis of all other components. One standard stock solution was made with 25 g/L of each of the sugars. The other standard stock solution contained succinic acid (10 g/L), L-lactic acid (12 g/L), acetic acid (12 g/L), *trans*-aconitic acid (5 g/L), glycerol (8 g/L), and ethanol (15 g/L). The stock solutions were taken out in glass vials and frozen. Vials were further thawed and 2, 4, 6, 8, and 10 µL was injected to generate calibration curves. All calibrations were found to be linear with very good correlations ($r^2 > 0.99$).

For one of the inhibitors studied (acetic acid), ethanol productivity was estimated from measured CO_2 production with assumption that ethanol and CO_2 are produced at the same molar rate during anaerobic growth phase (Atkinson and Mavituna 1991). Whenever CO_2 concentration is listed in this manuscript, it corresponds to the amount CO_2 produced divided by the liquid volume in the fermentation bottle.

At the end of the control experiment (containing activation medium), the broth contained on average ($n = 5$) 0.1, 0.2, 0.1, and 1.1 g/L of succinic acid, glycerol, acetic acid, and ethanol, respectively, and produced 0.2 g/L CO_2. The amount of ethanol in the control represent both ethanol production and minor amounts of ethanol from sanitation.

Results and Discussion

Baseline Fermentation

In Fig. 1, the typical timeline for the fermentation is shown for the synthetic medium containing sucrose, glucose, and

fructose as carbon sources. Sucrose is quickly converted into glucose and fructose by the yeast's invertase enzyme (Silveira et al. 2000) shortly after the fermentation is initiated, causing fructose and glucose concentration to increase and sucrose to disappear (see Fig. 1). Both the two C-6 sugars initially present (and generated from sucrose) are converted by the yeast concurrently (Fig. 1a). The major fermentation products for this particular yeast accumulate over time and were ethanol, glycerol, succinic acid, and carbon dioxide (Fig. 1b, c). While cell mass was also formed during the fermentation, the initial cell mass was large and increases were not statistically detectable; however, the increase in cell mass can be estimated from sugar consumption, product accumulation, and established relationships (Atkinson and Mavituna 1991). Typical cell loadings in commercial Brazilian yeast fermentation to produced ethanol biofuel from sugar are as high as 10–17 % [(wet cell weight (wcw) per volume] (Basso et al. 2008). Assuming a dry to wet biomass ratio of 0.32 (Govindaswamy and Vane 2007), the levels used here are lower (2.6 % wcw/v) but high enough to cause rapid fermentation and considerably higher than those used (0.025 % wcw/v) in dry grind corn ethanol plants (Kwiatkowski et al. 2006).

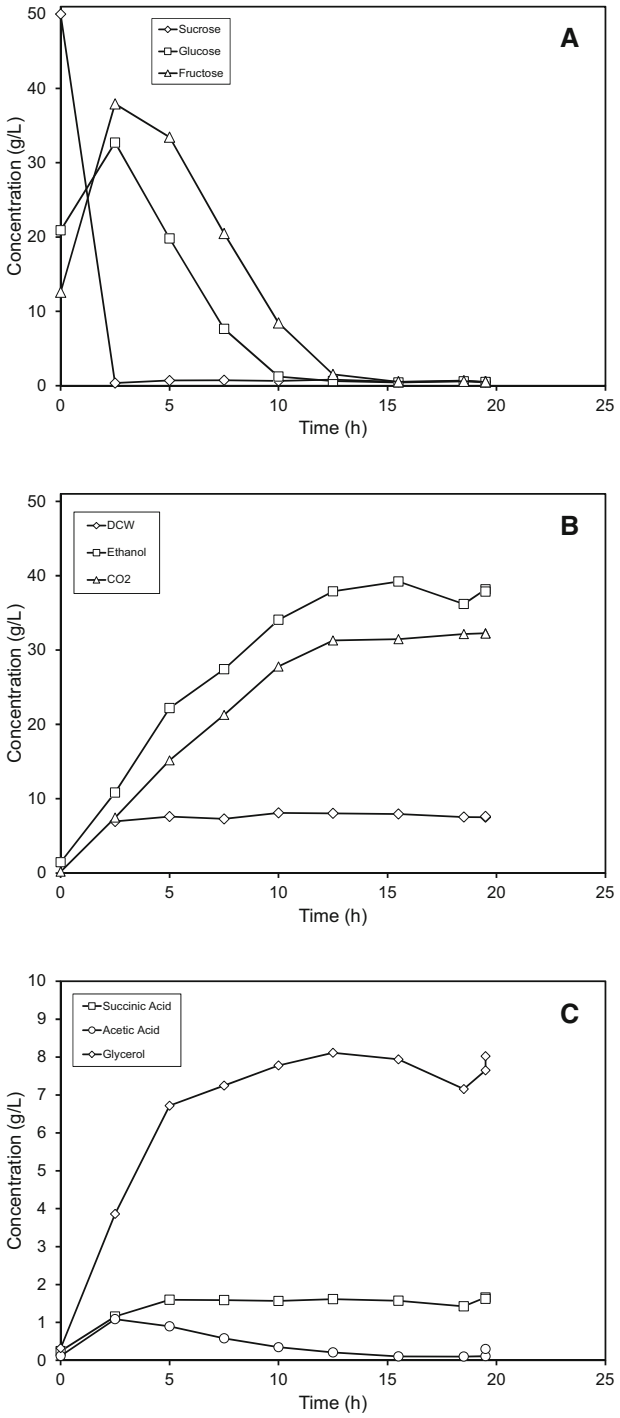
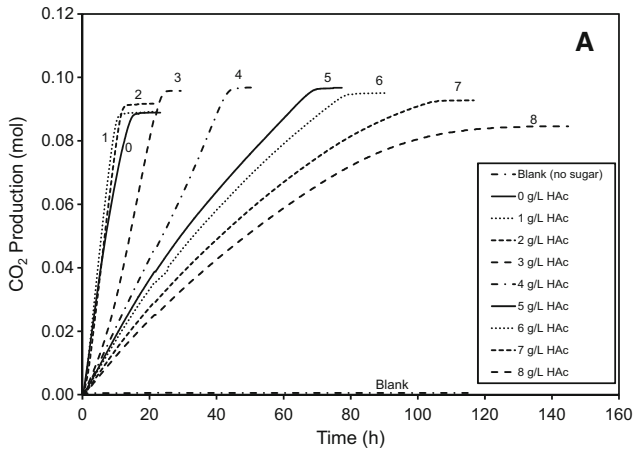
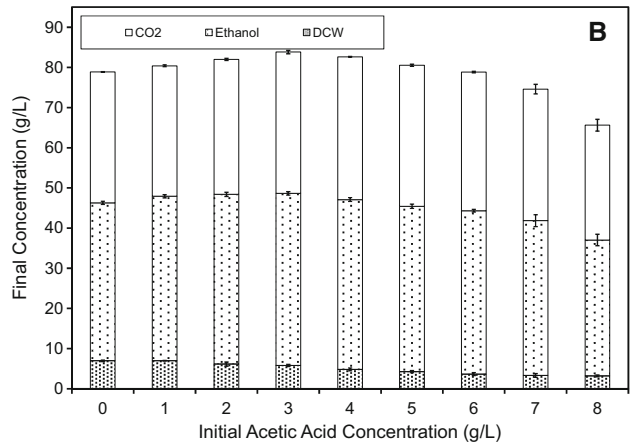


Fig. 1 Typical time line for fermentation of sweet sorghum juice sugars to ethanol

Impact of Acetic Acid

The impact of acetic acid on the fermentation was clear and expected. Fermentation progressed at all levels of added acetic acid (0–8 g/L) but at different rates as indicated by the CO_2 production (Fig. 2a). However, it was interesting to note that low levels of added acetic acid (1–2 g/L) improved the rate of CO_2 production. This improved rate, at low levels of acetic acid, has also been noted by others (Palmqvist et al. 1999; Pampulha and Loureiro-Dias 2000). Final ethanol and CO_2 production was reduced at the highest level (8 g/L) of initial acetic acid and appeared (but not statistically proven) promoted at some of lower concentrations, compared to control (Fig. 2b). This corresponded to reduced production of glycerol and succinic acids at the same concentrations (Fig. 2c), also recorded by Taherzadeh et al. (1997). As noted in Fig. 2a, the CO_2 production leveled off in all cases; however, fructose (3–10 g/L) was still available in the fermentation broth at the highest levels (7–8 g/L) of initial acetic acid (data not shown). In addition to the added acid, acetic acid and lactic acid were produced in the fermentation, especially at the higher levels of added acetic acid. For example, while 8 g/L of acetic acid was added, a total of 10.9 g/L of acetic acid was noted at the end of the fermentation. The rate of ethanol production (calculated from CO_2 production) improved with low levels (1–2 g/L) of acetic acid (Fig. 3). This is mainly attributed to the pH buffering impact of the organic acid (Fig. 3); without the addition of acetic acid, the final fermentation pH was approximately pH 3.1, which is less than the optimal pH 4–6 (Narendranath and Power 2005). With acetic acid addition, the final acidity was between pH 3.5 (with 1 g/L of added acetic acid) and pH 4.2 (with 3–8 g/L of added acetic acid).





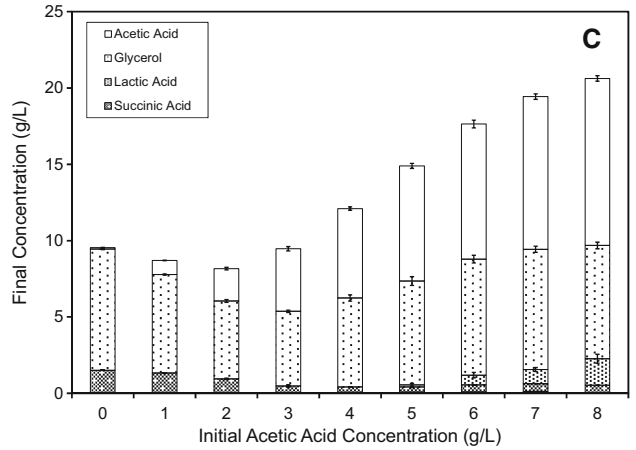


Fig. 2 Carbon dioxide production during a typical fermentation (from three conducted) and average ($n = 3$) final levels of products at the end of the fermentation, where different levels of acetic acid had been added at the start of the fermentation. *SE bars* are shown

The impact of acetic acid on ethanol fermentation is well known. Significant increases and decreases, as well as no impact, of ethanol production rates have been noted for different industrial and laboratory ethanol-producing yeast

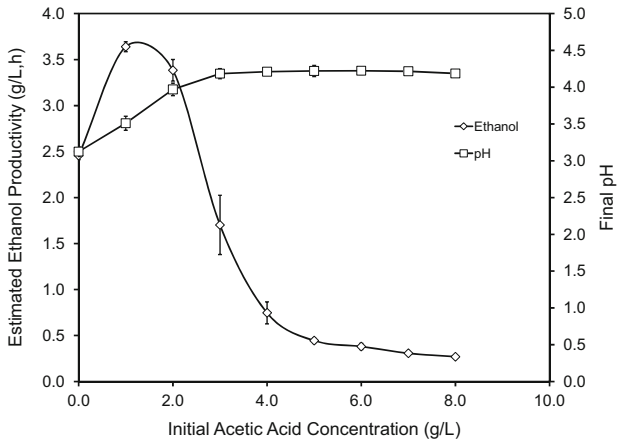


Fig. 3 Estimated ethanol productivity at mid-fermentation estimated from CO_2 production and the assumption that ethanol and CO_2 are produced at the same molar rate during anaerobic growth phase (Atkinson and Mavituna 1991). Ethanol fermentation efficiency is often estimated from CO_2 production (Amorim 2015). *SE bars* are shown ($n = 3$)

strains in the presence of acetic acid at 10 g/L (Garay-Arroyo et al. 2004). Others have noted complete inhibition of yeast cultures at 40 g/L of acetic acid (Graves et al. 2006). The undissociated form of acetic acid is considered as the influential species (Graves et al. 2006), and it has been shown that the growth of baker's yeast is prevented at 5 g/L of undissociated acetic acid (Taherzadeh et al. 1997). While growth rate was not directly measured in our experiments, the measured dry cell weight in fermentations containing acetic acid was lower than in the experiment with no acetic acid (Fig. 2b). CO_2 and ethanol production occurred at all the experimental conditions, which included undissociated acetic acid concentrations as high as 8.6 g/L (calculated from broth pH and pK_a 4.74) without statistically significant (Ryan's modified Q test, $\alpha = 0.05$, Day and Quinn 1989) reduction in actual ethanol yield (based on sugar consumed), compared to acetic acid-free controls.

Impact of Lactic Acid

Added lactic acid (1–5 g/L) had little impact on the fermentation of sugars to ethanol. The CO_2 production in each fermentation bottle can be seen in Fig. 4a, where the concentrations listed of lactic acid are nominal initial concentrations. The presence of lactic acid had a positive impact on the rate of CO_2 production. This was attributed to the pH buffering effect of lactic acid. This was also shown for acetic acid; however, the acetic acid became inhibitory at higher concentrations (see above section). In Fig. 4b, c, the fermentation products are shown. In these figures, we can see that none of the product levels were impacted by lactic acid in the medium. Considering that

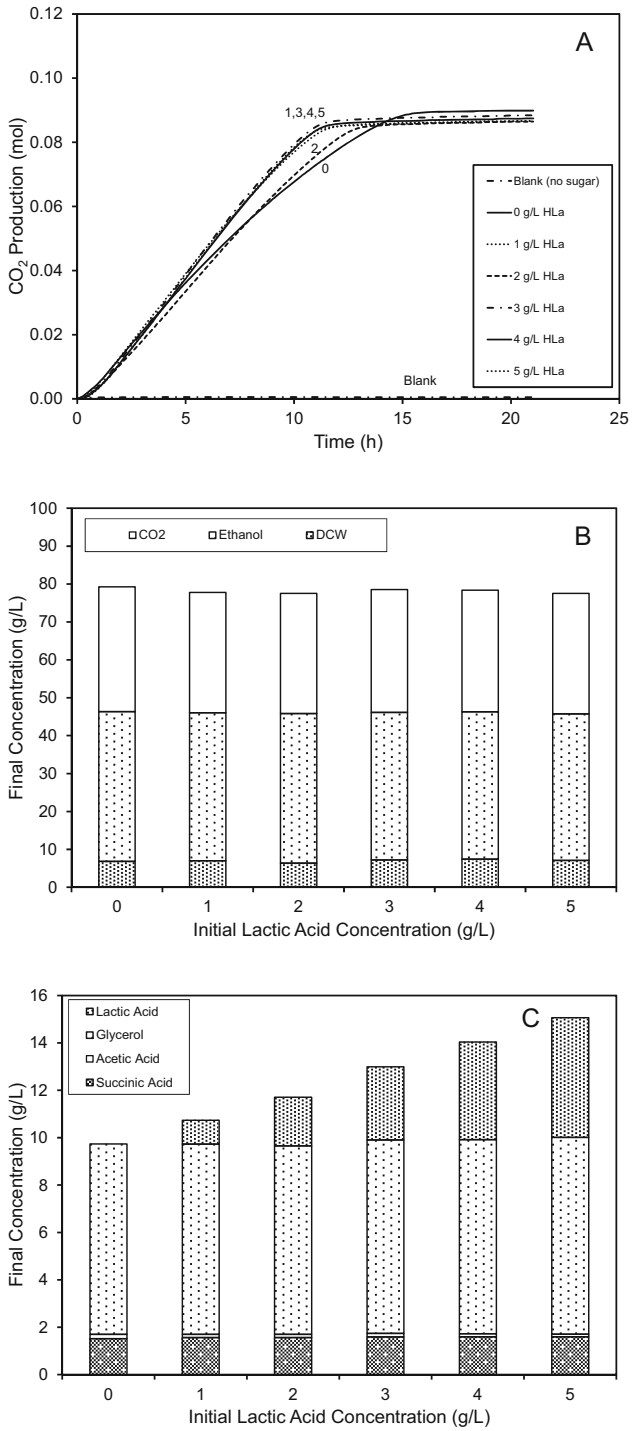


Fig. 4 Carbon dioxide production during fermentation and final levels of products at the end of the fermentation, where different levels of lactic acid had been added at the start of the fermentation

lactic acid is one of the most common products of bacterial contamination during storage of sweet sorghum juice (Kumar et al. 2013; Eggleston et al. 2015), it is valuable to know that that lactic acid is not detrimental to subsequent yeast fermentation. Higher levels of lactic acid were not studied because it is not anticipated that lactic acid will be present at high levels in sugar solutions from sweet sorghum. Decreased ethanol production from corn mash at 40 g/L of lactic acid was recorded at very high levels of lactic acid (Graves et al. 2006). In minimal medium, concentrations of 25 g/L of lactic acid were found inhibitory to yeast growth for two different yeast strains and lower concentrations (2–6 g/L) negatively impacted growth, sugar consumption, and ethanol production (Narendranath et al. 2001). Our results revealed no negative impact on yield and positive impact on rates at 5 g/L of lactic acid which indicated that yeast growth studies in minimal medium showed lower tolerance to organic acids than in complex medium (Narendranath et al. 2001). Others have also found that in complex media, the impact of lactic acid was negligible at 7 g/L (Savard et al. 2002). As previously mentioned, the inhibition of yeast by organic acids is tied to the concentration of the undissociated organic acid. At high levels of undissociated lactic acid, fermentation process have been found influenced (Essia Ngang et al. 1990); however, in these latter experiments, even the lowest level of undissociated lactic acid (4.1 g/L) was higher than the ones experienced in our studies, which were 0.8–2.4 g/L of undissociated lactic acid (pK_a 3.86).

Impact of Aconitic Acid

As in the case with lactic acid, added aconitic acid (1–5 g/L) had little impact on the fermentation of sugars to ethanol. The CO_2 production in each fermentation bottle can be seen in Fig. 5a, where the concentrations listed of aconitic acid are nominal initial concentration. The presence of aconitic acid had a positive impact on the rate of CO_2 production at all concentrations studied. We attribute that to the pH buffering effect of aconitic acid. Without aconitic acid, the final acidity of the fermentation was pH 3.0, and in the presence of aconitic acid, it was between pH 3.2 and pH 4.0. In Fig. 5b, c, the products of the fermentation are shown. As in the case of lactic acid, none of the product levels were impacted by aconitic acid in the medium. Higher concentrations than the levels shown were not used because it is not anticipated that aconitic acid will be present at high levels in juice from sweet sorghum (Amorim 2015). While no negative impact was noted on the ethanol production in the presence of aconitic acid, others have speculated that aconitic acid may have been the cause for lower fermentation efficiencies of concentrated sweet sorghum juices (Wu et al. 2010). However, Amorin (2015) reported that aconitic acid, at the low fermentation pH of 2.5 used in some of Brazil's ethanol plants, contributes to the decrease in CO_2 production rates (and thus ethanol production rates).

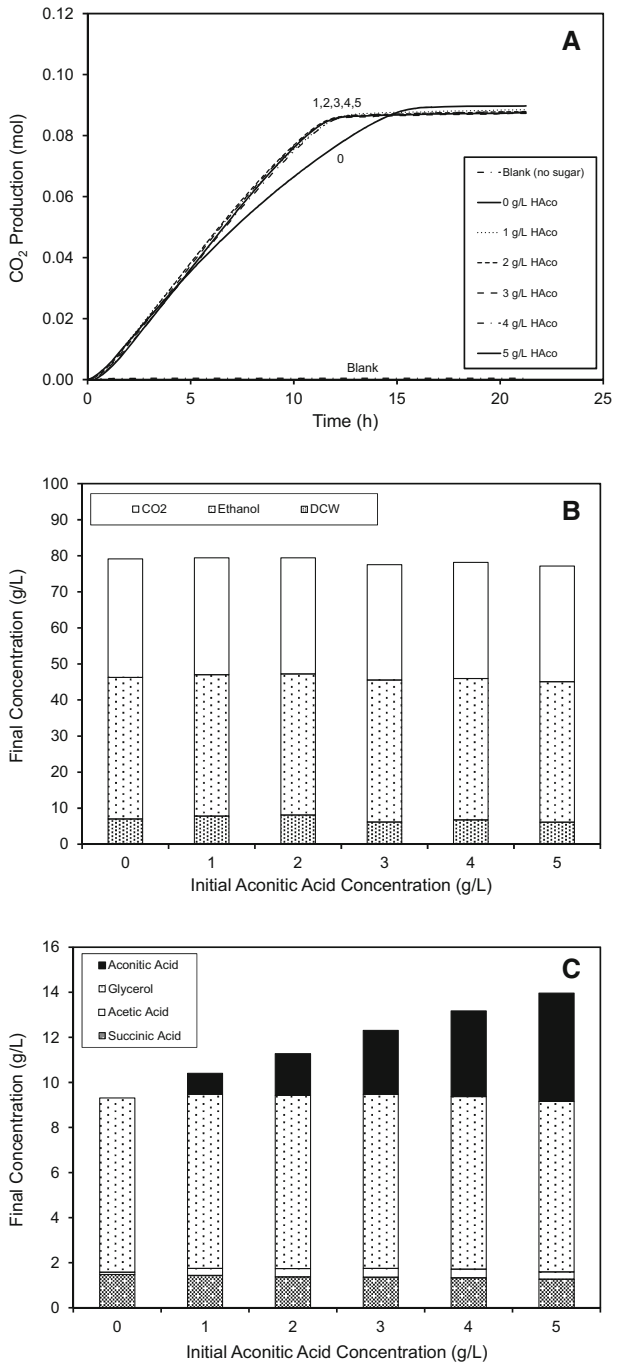


Fig. 5 Carbon dioxide production during fermentation and final levels of products at the end of the fermentation, where different levels of aconitic acid had been added at the start of the fermentation

Conclusions

Among the organic acids present in sweet sorghum juice fermentation, acetic acid had the most impact on fermentation of sweet sorghum sugars to ethanol by a commercial yeast strain. Fermentation rates were greater at low levels (1–2 g/L) of acetic acid, but average final ethanol concentrations were statistically the same below 8 g/L (added, 10.9 g/L final) of acetic acid. At the highest level of acetic acid of 8 g/L (added), the final ethanol concentrations were reduced compare to the other conditions and fructose conversion was found incomplete. Lactic acid and aconitic acid did not show the same negative impact on the fermentation. In fact, the presence of either lactic acid or aconitic acid showed positive impact in the fermentation rates. This was attributed to the pH buffering effect of the organic acids which prevented the pH to drop below pH 3.2. This positive impact on fermentation rate was also attributed to low acetic acid concentration which also has a pH buffering effect.

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Compliance with Ethical Standards

Conflict of interest Dr. Klasson has nothing to disclose.

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